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(54) **Title:** ENZYMES AS BIOMARKER FOR ALZHEIMER'S DISEASE

(57) **Abstract:** The present invention relates to a method for assessing Alzheimer's Disease in vitro comprising measuring in a body fluid sample the level of Peroxiredoxin 5 or Microsomal glutathione S-transferase 3 (MGST-3), or a variant thereof, wherein a decreased level of one of said proteins is indicative that said individual suffers from Alzheimer's Disease.

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Enzymes as biomarker for Alzheimer's Disease

Alzheimer's disease (AD) is a complex neurodegenerative dementing illness. It is histopathologically characterized by the deposition of extracellular amyloid plaques mainly consisting of amyloid-protein (Aβ) and the intracellular accumulation of hyperphosphorylated tau into neurofibrillary tangles.

In clinical practice, the diagnosis of AD is still largely based on cognitive tests and behavioral analysis. One of the most promising sources of biomarkers in AD is the cerebrospinal fluid (CSF). The CSF is, compared to the brain, more easily accessible and is in direct contact with the extracellular space of the central nervous system where biochemical changes in the brain could potentially be reflected. Therefore, a number of putative biological markers of AD have been suggested, like CSF total tau (Andreasen, N., Vanmechelen, E., Van de Voorde, A., Davidsson, P., Hesse, C., Tarvonen, S., Raiha, I., Sourander, L., Winblad, B., and Blennow, K. (1998) J Neurol Neurosurg Psychiatry 64, 298-305) and phospho-tau levels (Andreasen, N., Sjogren, M., and Blennow, K. (2003) World J Biol Psychiatry 4, 147-155), CSF A 1-42 levels(Ida, N., Hartmann, T., Pantel, J., Schroder, J., Zerfass, R., Forstl, H., Sandbrink, R., Masters, C. L., and Beyreuther, K. (1996) J Biol Chem 271, 22908-22914) or a combination thereof (Shoji, M., Matsubara, E., Kanai, M., Watanabe, M., Nakamura, T., Tomidokoro, Y., Shizuka, M., Wakabayashi, K., Igeta, Y., Ikeda, Y., Mizushima, K., Amari, M., Ishiguro, K., Kawarabayashi, T., Harigaya, Y., Okamoto, K., and Hirai, S. (1998) J Neurol Sci 158, 134-140). Yet, a definitive diagnosis of AD can still only be made by a postmortem assessment of brain neuropathology and there is clearly a need for a reliable biomarker for AD.

Therefore, the present invention provides a method for assessing Alzheimer's Disease in vitro comprising measuring in a body fluid sample the level of Peroxiredoxin 5 or Microsomal glutathione S-transferase 3 (MGST-3), or a variant thereof wherein a decreased level of one of said proteins is indicative that said individual suffers from Alzheimer's Disease.

Preferably, the protein of interest may be detected in the cerebrospinal fluid or blood.

The term "variants" in this context relates to proteins or peptides substantially similar to said proteins. The term "substantially similar" is well understood by the person

skilled in the art. In particular, a variant may be an isoform or allele which shows amino acid exchanges compared to the amino acid sequence of the most prevalent peptide isoform in the human population. Preferably, such a substantially similar peptide has a sequence similarity to the most prevalent isoform of the protein or peptide of at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95%. Substantially similar are also degradation products, e.g. proteolytic degradation products, which are still recognized by the diagnostic means or by ligands directed against the respective full-length protein or peptide. The term "variants" is also meant to relate to splice variants.

The term "variant" also relates to a post-translationally modified protein such as glycosylated protein. A "variant" is also a peptide which has been modified after collection of the sample, for example by covalent or non-covalent attachment of a label, particularly a radioactive or fluorescent label, to the protein.

The term "protein(s) of interest" as used herein refers to Peroxiredoxin 5 or Microsomal glutathione S-transferase 3 (MGST-3) or a variant thereof.

The invention also includes the measuring of different biomarkers in combination, simultaneously or non-simultaneously.

Peroxiredoxin 5 (SEQ. ID NO: 1) and MGST-3 (SEQ. ID NO: 2) are enzymes and are involved in the protection against oxidative stress. Oxidative stress refers to a state in which oxidant production surpasses the endogenous antioxidant capability leading to oxidative molecular damage of the tissue. Such a state can result from increased production of oxidants and/or decreased concentration of antioxidants. Stepwise reduction of oxygen during normal metabolism produces reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals.

The present invention further provides the use of Peroxiredoxin 5 or MGST-3, or a variant thereof as biomarker for Alzheimer's disease.

The term "biomarker" as used herein refers to molecules in an individual which are differentially present (i.e. present in increased or decreased levels) depending on presence or absence of a certain condition, disease, or complication. In particular, biochemical markers are gene expression products which are differentially present (e.g. through increased or decreased level of expression or turnover) in presence or absence of a certain condition, disease, or complication. A biomarker of the invention is biochemical marker. A biochemical marker is a protein, polypeptide or peptide. The level of a suitable biomarker can indicate the presence or absence of a particular condition, disease, or risk, and thus allow diagnosis or determination of the condition, disease or risk.

The person skilled in the art is familiar with different methods of measuring the level of a peptide or polypeptide. The term "level" relates to amount or concentration of a peptide or polypeptide in an individual or a sample taken from an individual.

In the context of the present invention, amount also relates to concentration. It is evident, that from the total amount of a substance of interest in a sample of known size, the concentration of the substance can be calculated, and vice versa.

The term "measuring" according to the present invention relates to determining the amount or concentration, preferably semi-quantitatively or quantitatively. Measuring can be done directly or indirectly. Indirect measuring includes measuring of cellular responses, bound ligands, labels, or enzymatic reaction products.

Measuring can be done according to any method known in the art. Preferred methods are described in the following

In a preferred embodiment, the method for measuring the level of a protein of interest, comprises the steps of (a) contacting a cell capable of a cellular response to the protein with the protein for an adequate period of time, (b) measuring the cellular response.

In another preferred embodiment, the method for measuring the level of a protein of interest comprises the steps of (a) contacting a protein with a specifically binding ligand, (b) (optionally) removing non-bound ligand, (c) measuring the amount of bound ligand.

In another preferred embodiment, the method for measuring the level of a protein, peptide or polypeptide of interest, comprises the steps of (a) contacting a protein, peptide or polypeptide with a suitable substrate for an adequate period of time, (b) measuring the amount of product.

Preferably, the protein is contained in a body fluid sample, and the amount of the protein in the sample is measured.

A body fluid may be blood, blood serum, blood plasma and cerebral liquor such as cerebrospinal fluid. Particularly, body fluids include cerebrospinal fluid. One important example is the measurement in CSF. Samples of body fluids can be obtained by any method known in the art.

If necessary, the samples may be further processed. Particularly, proteins may be purified from the sample according to methods known in the art, including filtration, centrifugation, or extraction methods such as chloroform/phenol extraction.

For measuring cellular responses, the sample or processed sample is added to a cell culture and an internal or external cellular response is measured. The cellular response may include the expression of a reporter gene or the secretion of a substance, e.g. a protein, peptide, polypeptide, or a small molecule.

Other preferred methods for measurement may include measuring the amount of a ligand binding specifically to the protein, peptide or polypeptide of interest. Binding according to the present invention includes both covalent and non-covalent binding.

A ligand according to the present invention can be any protein, peptide, polypeptide, nucleic acid, or other substance binding to the protein of interest. It is well known that proteins, if obtained or purified from the human or animal body, can be modified, e.g. by glycosylation. A suitable ligand according to the present invention may bind the protein exclusively or additionally via such sites.

Preferably, the ligand should bind specifically to the protein to be measured. "Specific binding" according to the present invention means that the ligand should not bind substantially to ("cross-react" with) another protein or substance present in the sample investigated. Preferably, the specifically bound protein or isoform should be bound with at least 3 times higher, more preferably at least 10 times higher and even more preferably at least 50 times higher affinity than any other relevant protein, peptide or polypeptide.

Non-specific binding may be tolerable, particularly if the investigated protein can still be distinguished and measured unequivocally, e.g. according to its size (such as on a Western Blot), or by its relatively higher abundance in the sample.

Binding of the ligand can be measured by any method known in the art. Preferably, the method is semi-quantitative or quantitative. Suitable methods are described in the following.

First, binding of a ligand may be measured directly, e.g. by NMR or surface plasmon resonance.

Second, the ligand may be coupled covalently or non-covalently to a label allowing detection and measurement of the ligand.

Labelling may be done by direct or indirect methods. Direct labelling involves coupling of the label directly (covalently or non-covalently) to the ligand. Indirect labelling involves binding (covalently or non-covalently) of a secondary ligand to the first ligand. The secondary ligand should specifically bind to the first ligand. Said secondary

ligand may be coupled with a suitable label and/or be the target (receptor) of tertiary ligand binding to the secondary ligand. The use of secondary, tertiary or even higher order ligands is often used to increase the signal. Suitable secondary and higher order ligands may include antibodies, secondary antibodies, and the well-known streptavidin-biotin system (Vector Laboratories, Inc.)

The ligand may also be "tagged" with one or more tags as known in the art.

Such tags may then be targets for higher order ligands. Suitable tags include biotin, digoxygenin, His-Tag, Glutathion-S-Transferase, FLAG, GFP, myc-tag, influenza A virus haemagglutinin (HA), maltose binding protein, and the like. In the case of a peptide or polypeptide, the tag is preferably at the N-terminus and/or C-terminus.

Suitable labels are any labels detectable by an appropriate detection method. Typical labels include gold particles, latex beads, acridan ester, luminol, ruthenium, enzymatically active labels, radioactive labels, magnetic labels ("e.g. magnetic beads", including paramagnetic and superparamagnetic labels), and fluorescent labels.

Enzymatically active labels include e.g. horseradish peroxidase, alkaline phosphatase, beta-Galactosidase, Luciferase, and derivatives thereof. Suitable substrates for detection include di-amino-benzidine (DAB), 3,3'-5,5'-tetramethylbenzidine, NBT-BCIP (4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, available as readymade stock solution from Roche Diagnostics), CDP-10 Star™ (Amersham Biosciences), ECF™ (Amersham Biosciences). A suitable enzyme-substrate combination may result in a colored reaction product, fluorescence or chemoluminescence, which can be measured according to methods known in the art (e.g. using a light-sensitive film or a suitable camera system).

Typical fluorescent labels include fluorescent proteins (such as GFP and its derivatives), Cy3, Cy5, Texas Red, Fluorescein, and the Alexa dyes (e.g. Alexa 568). Further fluorescent labels are available e.g. from Molecular Probes (Oregon). Also the use of quantum dots as fluorescent labels is contemplated.

Typical radioactive labels include 35S, 125I, 32P, 33P and the like. A radioactive label can be detected by any method known and appropriate, e.g. a light-sensitive film or a phosphor imager.

Suitable measurement methods according the present invention also include precipitation (particularly immunoprecipitation), electrochemiluminescence (electro-generated chemiluminescence), RIA (radioimmunoassay), ELISA (enzyme-linked immunosorbent assay), sandwich enzyme immune tests, electrochemiluminescence

sandwich immunoassays (ECLIA), dissociation-enhanced lanthanide fluoro immuno assay (DELFIA), scintillation proximity assay (SPA), turbidimetry, nephelometry, latexenhancedturbidimetry or nephelometry, solid phase immune tests, and mass spectrometry such as SELDI-TOF, MALDI-TOF, or capillary electrophoresis-mass spectrometry (CEMS).

Further methods known in the art (such as gel electrophoresis, 2D gel electrophoresis, SDS polyacrylamid gel electrophoresis (SDS-PAGE), Western Blotting), can be used alone or in combination with labelling or other detection methods as described above.

Preferred ligands include antibodies, nucleic acids, proteins, peptides or polypeptides, and aptamers, e.g. nucleic acid or peptide aptamers. Methods to such ligands are well-known in the art. For example, identification and production of suitable antibodies or aptamers is also offered by commercial suppliers. The person skilled in the art is familiar with methods to develop derivatives of such ligands with higher affinity or specificity. For example, random mutations can be introduced into the nucleic acids, proteins, peptides or polypeptides. These derivatives can then be tested for binding according to screening procedures known in the art, e.g. phage display.

Aptamers are chemically synthesized (usually short) strands of oligonucleotides (DNA or RNA) that can adopt highly specific three-dimensional conformations. Aptamers are designed to have appropriate binding affinities and specificities towards certain target molecules.

The term "antibody" as used herein includes both polyclonal and monoclonal antibodies, as well as any modifications or fragments thereof, such as Fv, Fab and F(ab)2 fragments that are capable of binding antigen or hapten.

The present invention also relates to a kit comprising a means or an agent for measuring Peroxiredoxin-5 or MGST-3, or a variant thereof.

Such means or agent may be any suitable means or agent known to the person skilled in the art. Examples for such means or agents as well as methods for their use have been given in this specification. For example, a suitable agent may be any kind of ligand or antibody specific for measuring said biomarkers. The kit may also comprise any other components deemed appropriate in the context of measuring the level(s) of the respective biomarkers, such as suitable buffers, filters, etc.

Optionally, the kit may additionally comprise a user's manual for interpreting the results of any measurement(s) with respect to determining whether an individual suffers

from Alzheimer's Disease. Particularly, such manual may include information about what measured level corresponds to a decreased level.

The present invention also relates to the use of said kit for assessing Alzheimer's Disease in an individual. The present invention also relates to the use of said kit in any of the methods according to the present invention for assessing Alzheimer's Disease in an individual.

The term "normal level" as used herein refers to the normal range of the level of protein of interest in a body fluid sample of a control. A control is one or more individuals not suffering from Alzheimer's Disease. Preferably, the number of individuals is higher than 100, more preferably more than 500, most preferably more than 1000. The normal range is determined by methods well known to the skilled person in the art. A preferred method is for example to determine the range of the values between quantile 2.5 and quantile 97.5, which leaves 5% of "normal" values outside the normal range or in other words, it covers 95% of all values of the control.

The pathological status is defied as deviation from the normal status. According to the invention this pathological status is indicated by a decreased or increased level of a biomarker. The term "decreased level" as used herein refers to the level of protein of interest in a body fluid sample which is significantly lower than the normal level. Significantly lower means that the level is lower and that the difference to the normal level is statistically relevant ($p \leq 0.05$, preferably, $p \leq 0.01$). The term "increased level" or "elevated level" as used herein refers to the level of protein of interest in a body fluid sample which is significantly higher than the normal level. Significantly higher means that the level is higher and that the difference to the normal level is statistically relevant ($p \leq 0.05$, preferably, $p \leq 0.01$).

The proteins of interest may also be used as target. Therefore, the present invention provides a method of screening for a compound which interacts with one of the proteins of the group consisting of Peroxiredoxin-5 and MGST-3, or a variant thereof. Such methods are well known in the art.

A suitable method is for example the method of screening for a compound which interacts with one of the proteins of the group consisting of Peroxiredoxin-5 or MGST-3, or a variant thereof comprising a) contacting one of said proteins with a compound or a plurality of compounds under conditions which allow interaction of said compound or a plurality of compounds with one of said proteins; and b) detecting the interaction between said compound or plurality of compounds with said protein.

The protein of interest may be immobilized prior step a) or between step a) and step b).

Having now generally described this invention, the same will become better understood by reference to the specific examples, which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified, in connection with the following figures.

Figures

Figure 1 shows an overview of the sample preparation procedure for LC/MS/MS analysis of brain tissue.

Figure 2 shows a SDS gel of solubilized protein sample of an AD case stained with Coomassie Blue. The sample lane was cut into 14 pieces that were subjected to trypsin digestion and LC/MS/MS analysis. The left lane shows the molecular weight marker, the gel excision pattern is shown on the right.

Examples

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated.

Example 1:

Materials and Methods

Human tissues - Human brains from AD patients were obtained from the Sun Health Research Institute through Dr. Thomas G. Beach (Sun City, Arizona, USA). The brain tissue was collected approximately two hours after death and was immediately frozen at -80°C. Brains were neuropathologically staged according to Braak and Braak (*Acta Neuropathol (Berl),* 1991, 82, 239-259). Brain tissue was isolated from the occipital association gyrus of 2 different AD patients (80 and 78 years old) with Braak stage VI and a control case (88 years old) with Braak stage II. The patient with Braak stage II showed no plaque load and was used as a control patient, the patients with Braak stage VI showed heavy plaque load.

Preparation of brain slices – Frozen brain tissue was used and sections of 30 µm thickness were cut with a cryostat microtome (Leica Microsystems, CM3050 S). The slices were directly mounted onto glass cover slip coated with a 1.35 µm thin polyethylene foil (PALM Robot Microbeam, LPC-MOMeNT-Object slides, 8150) and stored until staining at -20°C.

Sample preparation for LC-MS/MS – The brain tissue was isolated using a sterile surgical blade and measured areas of 30 and 35 mm2 (AD) and 32 and 36 mm2 (control) were collected. Exclusively brain tissue from grey matter areas was gathered. Brain tissue was solubilized by addition of 2x 50µl of 2% SDS, 66 mM Na2CO3, 2% β-mercapto-ethanol and 10% saccharose with intermediate heating for 10 min at 70°C. Supernatants were combined and protein was precipitated as previously described (Wessel, D., and Flugge, U. I. (1984) *Anal Biochem* 138, 141-143). Briefly, 400µl methanol were added to the supernatant and mixed thoroughly. 200µl of chloroform were added. After another mixing step and the addition of 300µl H2O, the sample was incubated on ice for 5 min and centrifuged at 10000 rpm for 2 minutes until a clear phase separation was achieved. The denatured proteins resided in the interphase and were isolated by carefully removing

the hydrophilic phase and the addition of 300µl methanol. After mixing and a second incubation on ice for 5 min, the proteins were sedimented by centrifugation for 5 min at 10000 rpm. The supernatant was removed and the protein pellet was dried at room temperature. Proteins in each sample were separated on a 4–12% SDS gel (1 mm thick) using 3-(N-morpholino) propanesulphonic acid (MOPS) running buffer for 1h 30 min at 120 volts. Subsequently, the gels were stained with Coomassie Blue G-250 overnight and decolorized for 6h in H2O. The entire lane was cut into 14 pieces followed by in-gel trypsin digestion. Several washing steps with 2 x 1 ml H2O, H2O / acetonitrile (ACN) (50% / 50%) and destainer solution (0.1M NH4HCO3 / ACN, 50% / 30% v/v) for 5 min each and incubation with 1 ml ACN preceded a reduction step with 0.1M NH4HCO3 / 10mM dithioerythritol for 45 min at 56°C. After two incubation steps with 1 ml ACN, the samples were alkylated with 0.1M NH4HCO3 / 55 mM iodoacetamide in the dark at room temperature for 30 min. The gel pieces were digested after two additional washing steps with NH4HCO3 and ACN by incubation with 30µl trypsin (0.02 µg / ml, Seq. Grade modified trypsin, Promega, Madison, WI, USA) overnight at room temperature. The peptides were eluted by addition of 250µl 25mM NH4HCO3 and 5% formic acid for 15 min at 37°C. The eluates were combined and evacuated to dryness.

Antibodies – The correlation of selected candidates detected by LC-MS/MS with immunohistochemistry or Western blotting was largely dependent on the availability of antibodies. As primary antibodies, the following were tested: anti-gelsolin (1:1000, Sigma, clone GS-2C4), anti-synaptotagmin (Synaptic Systems GmbH, Goettingen, Germany, clone 41.1), anti-synaptogyrin (Synaptic Systems, clone 80.1), anti-reticulon 1 (1:500, Santa Cruz Biotechnologies, Santa Cruz, CA, USA, clone Mon 160), anti-syntaxin (1:2000, Chemicon International, Temecula, CA, USA, clone MAB336), anti-Aβ (1:2000, The Genetics Company, Zuerich, Switzerland, clone WO-2), anti-ubiquitin (1:100, Dako, Glostrup, Denmark), anti-phosphotau (1:1000, kindly provided by Dr. F. Grüninger), anti-GFAP (1:2000, Accurate Chemical, Westbury, NY, USA, clone G-A-5), anti-Apolipoprotein E (1:2000, clone 3H1) and anti-ERK 1 / 2 (1:3000). As secondary antibodies, horseradish peroxidase linked anti-mouse or anti-rabbit antibodies were used (Amersham) for Western blot analysis.

Immunohistochemistry – Brain tissue slices were rehydrated for 5 min with phosphate buffered saline (PBS, Gibco, 14200-067) containing protease inhibitors (Roche Diagnostics GmbH, 1836145, 1 tablet per 50 ml) and fixed on the polyethylene foil covered glass slide by applying 70% acetone (0°C) for approximately 1 min. Sections were

washed twice for 2 min with 1 ml PBS and for doublestaining, thioflavine S was applied as a 1% aqueous solution for 3–5 min followed by a differentiation step with 70% ethanol for 3–5 min. By incubation of the sections with 500 µl PBS containing 1% bovine serum albumin (BSA, fraction V, Roche Applied Science) and 1% Ovalbumin (Sigma-Aldrich Chemie GmbH, 05440) for 15 min, unspecific binding sites were blocked. The slices were then incubated for 1 h at room temperature with 200 µl of 3 µg/ml primary antibody. The samples were then washed three times for 5 min with 500 µl of PBS containing 1% BSA. Detection was performed using either an Alexa Fluor 488 or 555-conjugated secondary antibody (secondary antibody diluted 1:200 in PBS containing 1% BSA; Invitrogen, Basel, Switzerland) applied for 1 h at room temperature. The preparations were then washed once for 5 min with 1 ml PBS and once for 2 min with distilled H2O before being air-dried and stored until submitted to fluorescence microscopy.

SDS-PAGE / Western blot – Frozen grey matter tissue from the corresponding brain areas were isolated after preparing 200 µm thick slices with a cryostat microtome using a surgical blade. The tissue was weighed and 4 µl of 2% SDS-buffer was added per mg brain. The brain tissue was homogenized using ceramic bead-containing tubes (MagNa Lyser Green Beads, 3 358 941, Roche Diagnostics GmbH, Mannheim, Germany) using a shaker (Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France) for 20 seconds. The supernatant was isolated and frozen at -20°C until analysis by Western blot. One µl of brain homogenate was separated on a 4–12% SDS gel (1 mm thick) using 2-(N-Morpholino)ethanesulfonic Acid (MES) running buffer for 1 h 15 min at 150 volts. Western blotting and immunostaining was performed as described by Wiltfang et al (*Electrophoresis,* 1997, 18, 527-532). Proteins were transferred for 60 min at 1 mA/cm2 and room temperature under semi-dry conditions onto Hybond membranes (Amersham Biosciences, Piscataway, NJ, USA). For immunostaining, membranes were air-dried for 15min and boiled for 5 min in PBS at 500 W using a microwave oven (Ida, N., Hartmann, T., Pantel, J., Schroder, J., Zerfass, R., Forstl, H., Sandbrink, R., Masters, C. L., and Beyreuther, K. (1996) *J Biol Chem* 271, 22908-22914; Wiltfang, J., Smirnov, A., Schnierstein, B., Kelemen, G., Matthies, U., Klafki, H. W., Staufenbiel, M., Huther, G., Ruther, E., and Kornhuber, J. (1997) *Electrophoresis* 18, 527-532). Blocking was performed for 1h at room temperature in the presence of 5% nonfat dry milk in PBS-T (PBS + 0.5% Tween-20) or Tris-Buffered Saline Tween-20 (TBS-T), respectively. Incubation with the primary antibodies was done overnight at 4°C. Membranes were then washed for 2 x 15 min, in PBS-T (or TBS-T). After the washing steps, the membranes were incubated with a horseradish peroxidase linked secondary antibody (Amersham) for 1h at room temperature. After 2 x 15 min washing in PBS-T (or TBS-T),

the membranes were developed for 2 min at room temperature with ECLTM (Amersham) solution according to the protocol of the manufacturer. Visualization was done with conventional X-Ray films (Amersham) and band intensities were compared. For the image analysis, GeneTools software (Syngene, Cambridge, United Kingdom) was used.

Confocal microscopy – Immunofluoroescence images were recorded on a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Glattbrugg, Switzerland). For the quantification of the intensity difference in AD and control samples, 10µm sections were immunolabeled with reticulon 1 antibody. For each case 3 images from grey matter were taken using a HCX PL APO CS 40x / 1.25 Oil objective. All instrument settings (illumination, PMT, magnification, speed) were selected to collect fluorescence signal in the effective dynamic range of the PMT and kept constant for a given protein to allow intensity comparison. Images were processed and analyzed using Imaris Software (Bitplane AG, Zuerich, Switzerland). Mean volume pixel intensity was determined for each case.

High resolution confocal immunofluoroescence images were recorded using HCX PL APO 63x / 1.3 Glyc Corr objective. The three dimensional image were reconstructed and processed using Imaris Software.

Analysis by LC-MS/MS – The resulting peptides from each gel piece were analyzed by liquid chromatography coupled to a LTQ ion trap mass spectrometer (Thermo Electronics, San Jose, CA USA) equipped with a nano-LC electrospray ionization source. Peptides were dissolved in 1% formic acid (buffer A: 0.5% Acetic acid, 0.012% Heptafluorobutyric acid) and concentrated and desalted online on a C18 PepMap 100 micro precolumn (5 µm particle size, 300 µm x 1 mm; Dionex Corporation, Sunnyvale, CA, USA) that was coupled to a self packed (7 cm, 3µm; ProntoSil C18-ACE-EPS, Bischoff Chromatography, Atlanta, GA, USA) and pulled (P-2000 laser puller, Sutter Instrument, Novato, CA, USA) fused silica capillary (100 µm i.d. × 365 µm o.d.). The chromatographic separation was then performed by a 100 minute nonlinear gradient from 5 to 55% buffer B (80% ACN/0.5% Acetic Acid/0.012% HFBA) with a constant flow rate of 0.20µl/min.

The mass spectrometric data acquisition was performed with a survey scan followed by 7 data dependent MS2 scans with a repeat count of 2. The collision energy was set to 35%.

Database searching for protein identification – SEQUEST was used to for searching the HumanGP database for peptide sequence and protein identification. HumanGP is a protein sequence database that is derived by assembling in sequences the results of Blast searches against the human chromosomes of a non-redundant protein set from Swissprot and Trembl (from Human, Mouse and other major organisms). Search parameters included differential mass modification to methionine due to possible oxidation and static mass modification to cysteine due to alkylation by iodoacetamide. Furthermore one missed cleavage of trypsin was accepted.

Protein quantification by mass spectrometry – Protein (semi-)quantification was based on the comparison of peptide counts, which reflect the number of peptides identified for a given protein, in samples derived from AD patients with samples from age-matched controls. This number is indicative for the relative abundance of a protein in a sample. The comparison was performed with in-house developed software (MSPresso).

Criteria for peptide and protein identification – For the comparisons of peptide counts, the single datasets from the analysis of the respective samples were combined to final sets.

Peptides identified by SEQUEST may have three different charge states (+1, +2, or +3), each of which results in a unique spectrum for the same peptide. Except in rare instances, an accepted SEQUEST result had to have a ΔCn score of at least 0.1 (regardless of charge state). Peptides with a +1 charge state were accepted if they were fully tryptic or the C-terminus of a protein and had a cross correlation (Xcorr) of at least 1.8. Peptides with a +2 charge state were accepted if they were fully tryptic or partially tryptic between the Xcorr ranges of at least 2.3 and higher. Finally, +3 peptides were only accepted if they were fully or partially tryptic and had an

Xcorr > 2.8. Only proteins identified with at least 2 different peptides, with a pepcount (number of identified peptides) larger than 7 were taken into account. In addition, only proteins identified in both of the two independent datasets of AD versus healthy, were accepted.

Results

Comparison of protein levels in AD brain and control – To compare protein levels from an AD brain with an control, two comparable sized brain areas from grey matter of AD patients with Braak stage VI were isolated and finally submitted to LC-MS/MS.

As the brain tissue was cut out using a surgical blade, it was not possible to dissect exactly the same size of brain areas. To overcome this limitation, the peptide counts of the AD patients were normalized. For this reason, the ratios of several abundant proteins (tubulin, actin, actinin and GAPDH) in the samples were averaged and the peptide counts in the AD patients were standardized against this ratio. Therefore, the peptide counts of the first AD patient were normalized with 0.93, the second AD patient with 1.44. The comparison of protein levels was based on the normalized peptide counts.

Next, only proteins, where the measured peptide ratio in the respective pair of samples was in a range of ± 0.3, were accepted. For example, protein X was found in the first measurement with a ratio of 0.6 between AD patient and control and in the second series with a ratio of 0.8. In this case, the average was taken (0.7) and the protein was accepted for the analysis and evaluation. Some exceptions were made, but only when the ratios belonged into the same classification group (see below). Proteins consistently present in only the AD or the control sample, were classified as well.

To evaluate the proteins with significantly altered protein levels in AD brain samples, they were accessed into functional groups. As the number of proteins detected with this method was more than 2000, the protein sets had to be screened for proteins related to the functional groups mentioned above. Ubiquitous proteins with comparable protein levels were not considered as important and were therefore not listed.

The peptide count is indicative for protein levels actually present in the brain. The peptide count reflects the number of peptides identified for a given protein after said protein was digested with an enzyme. As not all peptides produced by the digestion may be measured, the approach using peptide count is not quantitative, but semi quantitative, therefore it is only possible to describe a tendency and to assign the proteins into classification groups.

A protein with an averaged peptide count from the two series of $1.3 < x$ was considered to be increased. A protein with an averaged ratio of $0.7 < x < 1.3$ was regarded as comparable, whereas a ratio being $x < 0.7$ was accepted as decreased.

Using this approach, a large number of previously identified proteins related to AD could be confirmed in this report and demonstrate the feasibility of the approach. Among

these proteins, we found increased levels of Aβ, tau, apolipoprotein E, Ubiquitin, several heat shock proteins (e.g. Hsp90), members of the complement pathway (complement C4 and C3), as well as GFAP. Other proteins known to be associated with AD (e.g. antichymotrypsin, serum amyloid P component or C-reactive protein) were detected, but due to their low abundance / accessibility or unfavorable ionization / flight properties in the LC-MS/MS approach only in one AD patient or with peptide counts below 8 and were therefore discarded.

Two enzymes were not reported before in relation to Alzheimer's Disease and showed significantly decreased levels of expression in the AD patients. These proteins were Peroxiredoxin and MGST-3.

Protein Name	AD	AMC	ratio	r/0.93		AD	AMC	ratio	r/1.44		av.
Peroxiredoxin 5	89	145	0.61	0.66		181	213	0.85	0.59		0.63
Microsomal glutathione S-transferase 3 (MGST-3)	29	53	0.55	0.59		47	90	0.52	0.36		0.48

Table 1: Peptide counts of the proteins of invention. The peptide counts were normalized: with 0.93 for the peptide counts of the first AD-patient (white) and with 1.44 for the peptide counts of the second AD-patient (grey). Average peptide counts of $x < 0.7$ means that the expression level in AD patient is decreased compared to a control. AD = Patient suffering from Alzheimer's Disease, AMC = Age matched control (=one or more individual who have a similar age as the individual suffering from Alzheimer's Disease), r= range: ± 0.3, av. = average of the two patients.

Claims

1. A method for assessing Alzheimer's Disease in vitro comprising measuring in a body fluid sample the level of Peroxiredoxin 5 or Microsomal glutathione S-transferase 3 (MGST-3), or a variant thereof, wherein a decreased level of one of said proteins is indicative that said individual suffers from Alzheimer's Disease.

2. The method according to claim 1 wherein the body fluid is cerebrospinal fluid or blood.

3. Use of Peroxiredoxin 5, Microsomal glutathione S-transferase 3 (MGST-3), or a variant thereof, as biomarker for Alzheimer's Disease.

4. A kit comprising a means or an agent for measuring of Peroxiredoxin 5 or Microsomal glutathione S-transferase 3 (MGST-3), or a variant thereof.

5. The kit according to claim 4 wherein the kit further comprises a user's manual for interpreting the results of any measurement with respect to determining the risk of an individual suffering from Alzheimer's Disease.

6. Use of a kit according to claim 4 or 5 for determining the risk of an individual of suffering from Alzheimer's Disease.

7. Use of a kit according to claim 4 or 5 in a method according to any of claims 1 to 2.

8. Methods, uses and kits substantially as herein before described especially with reference to the foregoing examples

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Figure1

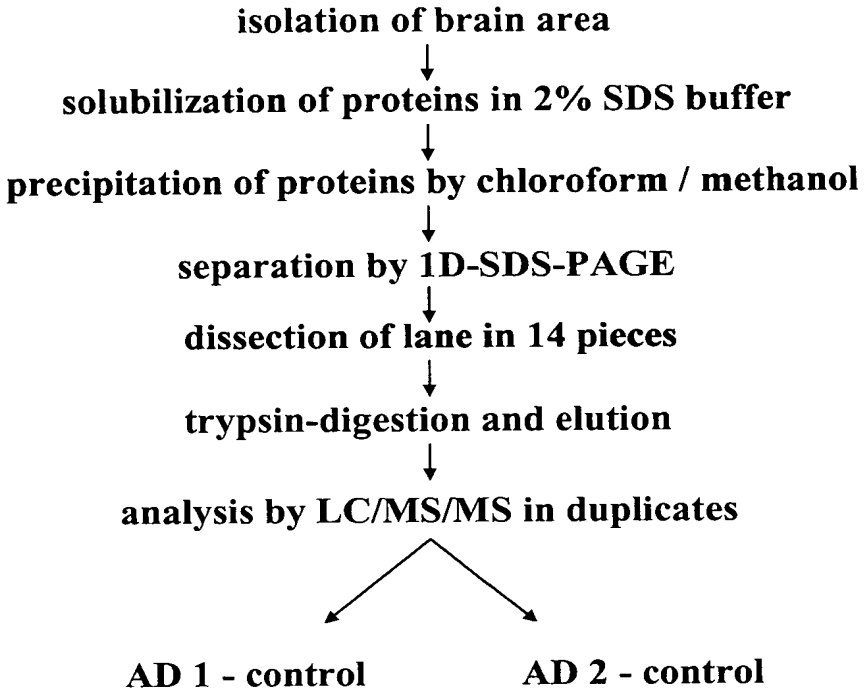

Figure 2